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Regulating effects of insertion direction of matrix attachment regions on transgenic expression in stably transformed Chinese hamster ovary cells

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ABSTRACT. We investigated the effects of different directions of insertion of matrix attachment region (MAR) sequences on transgenic expression in stably transformed Chinese hamster ovary (CHO) cells. The MAR sequences were inserted in forward or reverse directions into the expression vectors, and transfected into CHO cells. The expression of the chloramphenicol acetyltransferase (*CAT*) reporter gene and the relative copy numbers of the *CAT* gene were analyzed. The *CAT* gene expression levels in the vector with the MAR sequence inserted in the forward or reverse directions increased compared with expression without the MAR sequence. The relative copy numbers of the *CAT* gene with MAR sequenced vectors inserted in the reverse and forward directions were lower, than in the control group. The direction of insertion of MAR sequences had no significant effect on expression levels. The expression levels were not proportional to the copy numbers

of the gene.

Key words: Directional effect; Matrix attachment region; Transgenic copy number; Reporter gene

INTRODUCTION

Issues about transgenic expression efficiency and stability have increased research interest in matrix attachment regions (MARs). Studies have shown that using MARs effectively addresses these transgenic expression issues. MARs are A/T-rich DNA sequences in eukaryotic chromatin with lengths ranging from 300 to 1000 bp. They, bind to the nuclear matrix (Wang et al., 2008). A number of studies have shown that the MAR sequence can be cloned into an expression vector and transformed to allow the organism to overcome transgene silencing, improve the level of transgene expression, and reduce the differences in expression among transformants (Vain et al., 1999). Studies have confirmed that the effects on transgene expression of MAR sequences from different sources are not consistent (Neznanov et al., 1996; Cheng et al., 2001; Xu et al., 2011; Xue et al., 2012; Geng et al., 2013). The β-interferon scaffold attachment region confers high-level transgene expression and avoids extinction by the epigenetic modifications of an integrated provirus in adipose tissue-derived human mesenchymal stem cells (Moreno et al., 2011).

Our previous studies have confirmed that MAR sequences can increase the transgenic expression levels of the chloramphenicol acetyltransferase (*CAT*) reporter gene in Chinese hamster ovary (CHO) cells, and that heterologous MAR sequences in the sides of the expression cassette more effectively improve transgenic expression than homologous MAR sequences (Wang et al., 2012). However, few studies have investigated whether the increase in the expression level of the *CAT* gene caused by MAR sequences in CHO cells is related to the direction of insertion. In the present study, 5' end sequences with an eukaryotic expression vector containing an expression cassette with reverse-insertion globin-MAR were constructed. The effects on transgenic expression of inserting MAR sequences into one side of the expression cassette from different directions were analyzed, and the regulatory functions of MARs were studied further.

MATERIAL AND METHODS

Construction of the vector with MAR sequences inserted in the reverse direction

Based on human β-globin MAR sequences stored in GenBank (Accession No: L22754), the primers were designed as follows: 5'-TTAGTAAGACATCACCTTGCATTT-3'(forward), 5'-AGCCATAGTTTGAGTTACCCTTT-3' (reverse). To achieve directional cloning, *Bgl*II/*Kpn*I restriction sites (shown in bold) (AGC**GGTACC**, GTC**AGACTC**) were designed into the 5' ends of the primers. Using genomic DNA extracted from human blood as templates, the polymerase chain reaction (PCR) amplification parameters were as follows: 95°C for 3 min; 94°C for 40 s; 60°-56°C for 40 s; 72°C for 40 s, 30 cycles; and 72°C for 3 min. The PCR amplification products were analyzed by 2% agarose gel electrophoresis, recycled, ligated to the pMD-18T vector, and transformed. Positive clones were extracted, identified by electrophoresis, and sequenced.

The globin-MAR fragments connected to the T-vector were digested using *Bgl*II/*Kpn*I

restriction endonucleases. The vector pCAG (from our laboratory; Wang et al., 2010) was digested using the same enzymes. The digestion system was as follows: 10 µL MAR or pCAG, 0.5 µL *Kpn*I (15 U/µL), 0.5 µL *Bgl*II (15 U/µL), and 2 µL 10X T buffer. Afterward, 7.5 µL double-distilled water was added, and the system was left in a water bath at 37°C for 3 h, then analyzed by 1% agarose gel electrophoresis. The β-globin MAR fragments were recycled and the plasmid pCAG was linearized. The fragments and plasmid were then ligated and transformed. Single colonies were selected for subculturing. The plasmid was digested using restriction enzymes *Kpn*I/*Bgl*II and *Kpn*I. The correct plasmid obtained in the digestion test was verified by means of sequencing (Figure 1). The expression vector with MAR sequences inserted in the forward direction had been constructed previously (Wang et al., 2010). All the enzymes and the pMD-18T vectors were purchased from Takara (Dalian, China).

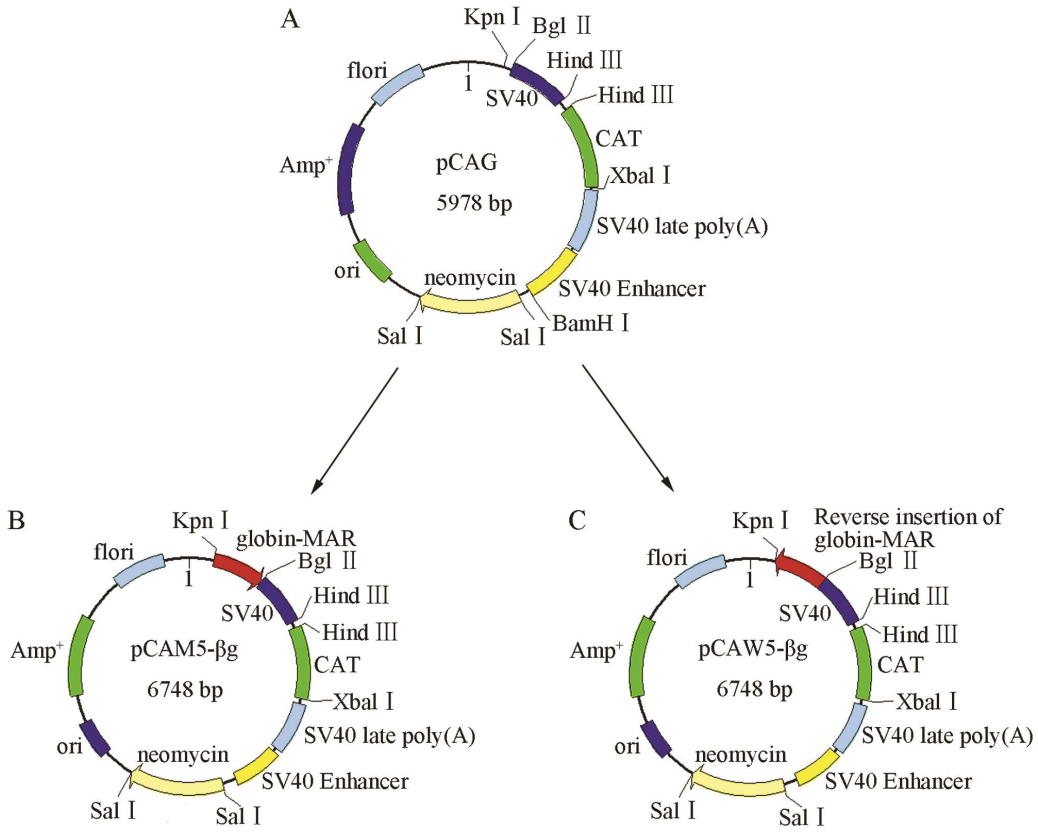

Figure 1. Vector construction **A:** pCAG (no MAR sequences in the expression cassette sides). **B:** pCCM5-βg (expression cassette 5' end containing the β-globin MAR sequences inserted in the forward direction). **C:** pCAW5-βg (expression cassette 5' end containing the β-globin MAR sequence inserted in the reverse direction).

Cell culture and transfection

CHO cells (Chinese Society of Laboratory Animal Research Institute) were cultured

using Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA), which contains 10% heat-inactivated fetal bovine serum (Sijiqing, China), 20 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. The cells were cultured at 37°C in 5% CO_2. The cells were transfected from the 24-well culture plates when cell density reached 8 x 10^4-2.0 x 10^5 cells/plate. The cells were transfected using Suohua-sofast™ (Sunmabio, Xiamen, China) reagents. The serum-free medium was removed 6 h after transfection and replaced with DMEM containing 10% fetal bovine serum for further incubation. This medium was replaced with 600 μg/mL G418 (Sangon, Shanghai, China) in DMEM 24 h after transfection, then cells were selected for 2-3 weeks for screening. The transfected cells were diluted and monocloned, then distributed on 96-well culture plates for sequential culturing. The G418 concentration was decreased to 200 μg/mL. After culturing for approximately 12 days, monoclonal colonies were collected and digested using 0.25% trypsin. The cells were then transferred to culture flasks for further analysis.

CAT detection

The cells were collected when the cell density in the culture flasks reached 1.0 x 10^6 cells/mL, and washed with phosphate-buffered saline. Approximately 1 mL lysis buffer was added to the tube in which the cell lysate was incubated at room temperature for 30 min (15°-25°C). The cell lysate was centrifuged at 4°C and 15,000 *g* for 10 min. The supernatant CAT enzyme levels were measured using a CAT enzyme-linked immunosorbent assay kit (Roche, Nutley, NJ, USA). The experiment was repeated three times independently.

Real-time PCR

The relative copy numbers of the *CAT* gene were detected using real-time quantitative PCR with SYBR PCR Master Mix reagents (Beijing Kang Biotechnology, Beijing, China) and an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Genomic DNA was extracted from 2.0 x 10^6 to 5.0 x 10^6 cells using a genomic extraction kit (Beijing Kang Biotechnology, Beijing, China). The *CAT* gene amplification primers were designed as follows: 5'-CATCGCTCTGGAGTGAATACC-3' (forward), 5'-GGCATCAGCACCTT GTCG-3' (reverse). Primers 5'-GTCTTTCTTCTGCCGTTCTC -3' and 5'-ACCAG CCTCAT TAGGTTTGT-3' were used to amplify β-actin, which was taken as an internal reference gene. The PCR regimen was as follows: 95°C for 10 min; 95°C for 15 s; and 60°C for 1 min, for 40 cycles. The plasmid with correct sequences was serially diluted to yield a quantitative standard amplification curve. All experiments were repeated three times. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative change in gene expression levels (Livak and Schmittgen, 2001).

RESULTS

Vector construction

T-vectors ligated with globin-MAR fragments and pCAG plasmids were digested using *Kpn*I/*Bgl*II restriction enzymes, and ligated with the recovered MAR fragment to the pCAG linear plasmid after digestion. The plasmids were extracted and digested using *Kpn*I/*Bgl*II and *Kpn*I. The constructed plasmid had a fragment of 770 bp cut out after *Kpn*I/*Bgl*II

digestion, and displayed a single linear DNA after digestion by a single enzyme, *Kpn*I. The digestion results matched the construction results exactly (Figure 2), and were then delivered to the sequencing company. Correct sequences indicated that an expression vector pCAW5-βg with globin-MAR sequences inserted in a reverse direction into the 5' end of the expression cassette had been successfully constructed.

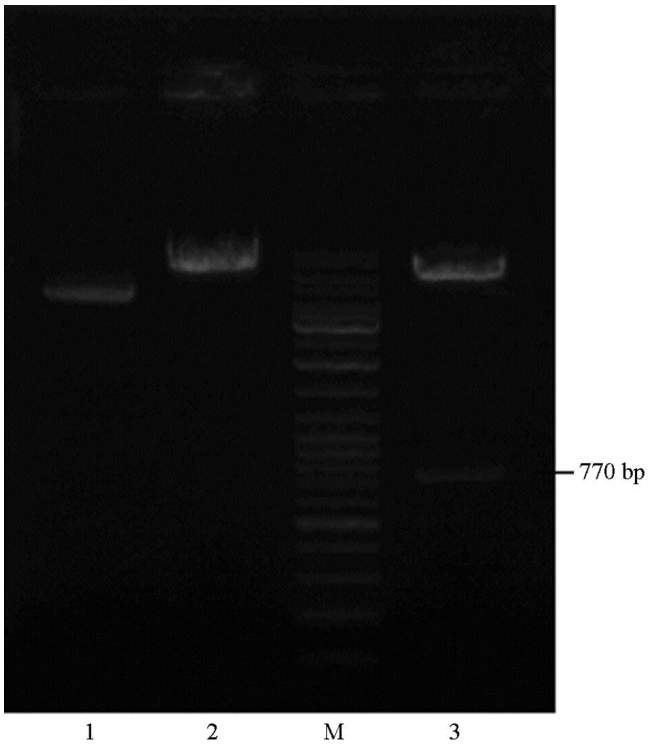

Figure 2. pCAW5-βg digestion scheme. *Lane 1* = No digested plasmid; *lane 2* = *Kpn*I single enzyme digestion; *lane M* = DNA marker; *lane 3* = *Kpn*I/*Bgl*II double enzyme digestion.

CAT enzyme content analysis

The CHO cells were transformed using different constructed expression vectors. A pCAG empty plasmid without MAR sequences was used as a control for the CAT activity analysis. The results of the statistical analysis of the 10 single cell clones selected showed that the expression level of the expression vector pCAW5-βg *CAT* gene with an expression cassette at the 5' end containing globin-MAR sequences inserted in a reverse direction increased 2.1 times compared with that of the control plasmid pCAG (expression cassette at the 5' end without MAR sequences), and decreased by 0.9 times compared with that of the vector pCCM5-βg with an expression cassette 5' end sequence inserted in a forward direction with globin-MAR. The expression level of the pCCM5-βg *CAT* gene increased 2.4 times compared with that of the control plasmid pCAG. Although the CAT enzyme content of the cells transformed by vector pCAW5-βg and pCCM5-βg increased, unlike that of the control vector pCAG, no significant difference was observed between the two vectors (Figure 3 and Table 1).

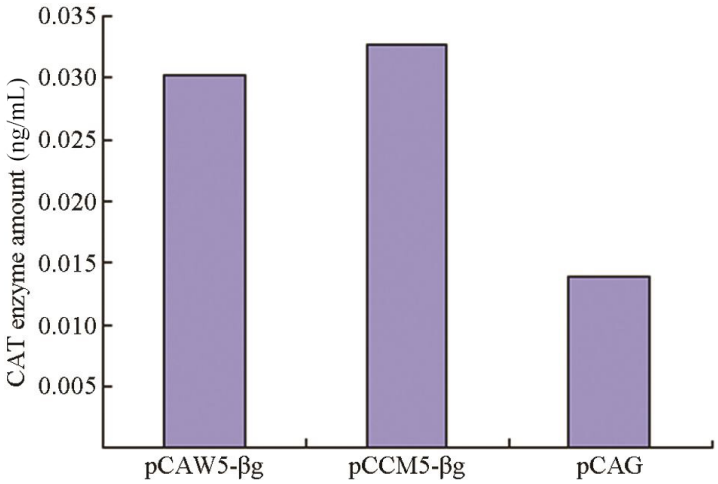

Figure 3. Effect of MAR on stable expression of the *CAT* gene. Average CAT enzyme content of different vector-transformed cells.

Table 1. Statistical comparison of CAT gene expressions in CHO cells transfected using different vectors.

Statistic	pCAW5-βg	pCCM5-βg	pCAG
Sample (N)	10	10	10
Mean expression	0.03	0.033	0.014
SD	0.008	0.025	0.015
CV	0.267	0.756	1.113
Fold-change	2.1*	2.4*	-

*P < 0.01, compared with pCAG.

Copy number analysis

The relative copy numbers of the *CAT* gene in the transfected CHO cells were analyzed using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. The relative copy numbers of the *CAT* gene in the CHO cells transfected by pCAW5-βg increased 1.028 times more than those transfected by pCCM5-βg ($P < 0.01$), and decreased 0.835 times more than those of pCAG. However, the relative copy numbers of pCAW5-βg were 0.812 times lower than those of the control vector pCAG ($P < 0.01$). These results indicate that the CAT protein concentrations were not directly proportional to the gene copy numbers in the CHO cells (Table 2).

Table 2. Comparison of relative gene copy numbers in CHO cells transfected using different vectors.

	C_T Mean (Vector)	C_T Mean (β-actin)	ΔC_T Mean	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
pCAW5-βg	30.15	25.90	4.25	0	1
pCCM5-βg	30.18	25.89	4.29	-0.04	1.028
pCAG	27.20	23.21	3.99	0.26	-0.835

DISCUSSION

Given that eukaryotic mammalian cell expression systems can accomplish correct protein folding and posttranslational processing of foreign genes, they are important to genetic

engineering. However, the expression level of foreign genes is not high because of gene silencing caused by factors such as position effects. Thus, increasing the expression level of foreign genes and overcoming gene silencing are necessary. The use of the MAR, developed in recent years, can effectively overcome exogenous gene inactivation. Many studies have focused on increasing the expression of exogenous genes using nuclear MARs (Klehr et al., 1991; Chavali et al., 2011; Harraghy et al., 2011; Jin et al., 2012; Koirala et al., 2013; Ley et al., 2013).

MAR sequences may increase the production of recombinant proteins (Harraghy et al., 2012), and β-globin MAR can improve the stable genomic expression of the Sleeping Beauty transposon (Sjeklocha et al., 2011). The development of S/MAR minicircles can enhance and assist transgene expression in the mouse liver (Argyros et al., 2011). In the present study, two constructed eukaryotic expression vectors, each with an expression cassette 5' end containing MAR sequences, one inserted in the forward direction and the other in the reverse direction, were used to transfect CHO cells. The results show that the *CAT* enzyme content of the cells transformed by globin-MAR sequences inserted in reverse and forward directions improved significantly compared with those of the cells transformed without MAR sequences. However, the expression levels of the *CAT* reporter genes of the two cells exhibited no significant differences. This effect may be due to the particular base sequences formed by the MAR fragments and the DNA bases, which are not affected by DNA direction. These findings are consistent with those of previous studies (Zahn-Zabal et al., 2001).

Studies have shown that the transgenic expression functions of MARs are unrelated to transgenic copy numbers, and the presence of MARs has no effect on transgenic copy numbers (Piechaczek et al., 1999; Li et al., 2008; Wang et al., 2010, 2012). However, some studies have suggested that the effect of MARs on transgenic expression depends on transgenic copy numbers (Kim et al., 2004; Oh et al., 2005). Our results indicate that the expression level of the MAR *CAT* gene inserted in the reverse direction is lower than that of the MAR inserted in the forward direction. However, the relative gene copy numbers of the reverse MAR were higher than those of the forward MAR. Thus, transgenic expression levels and gene copy numbers are not directly related to the direction of insertion of MARs.

Although numerous experiments have proven that MAR sequences can improve exogenous gene expression levels and reduce transgenic silencing, the gene expression regulation of MARs at the chromatin level and the mechanisms of transgenic expression effects are still poorly understood and require further in-depth study.

Conflicts of interest

The authors declare no conflict of interest.

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